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Phytodecta fornicata Brüggemann resistance mediated by oryzacystatin II proteinase inhibitor transgene

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Abstract Phytodecta fornicata Brüggemann is a serious pest of alfalfa (Medicago sativa L.) that causes significant crop loss in the Balkan peninsula of Europe. We introduced a wound-inducible oryzacystatin II (OCII) gene to alfalfa to evaluate its effect on survival of *P. fornicata* larvae. Feeding bioassays with second, third and fourth instars were carried out using transgenic plants that were shown to express OCII at 24 and 48 h after wounding. Second and third instars were the most sensitive to the ingestion of OCII, whereas no effects were observed with fourth instars. About 80% of the second and third instars died after 2 days of feeding on the transgenic plants as compared to 0-40% on the controls. This is the first report that demonstrates significant increase in mortality of P. fornicata on transgenic plants that express a cysteine proteinase inhibitor gene, and this knowledge should lead to the development of effective management strategies for this devastating pest of alfalfa.

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Introduction

Transformation of plant genomes with reconstructed proteinase inhibitor (PI) genes has been shown to enhance plant resistance to insects (Leple et al. 1995; Xu et al. 1996). PIs are proteins that occur naturally in a number of plant species and are characterized by varied specificity toward proteases, among them digestive proteases (Abe and Arai 1985; Brzin et al. 1998). Digestive proteases belong to one of four groups based on the amino acid residue or metal ion involved in peptide bond catalysis (Barrett 1986): (i) serine, (ii) cysteine (or thiol), (iii) aspartyl (or carboxyl), and (iv) metalloproteases (Applebaum 1985). Serine proteases have been found predominantly in midguts of lepidopteran and dipteran insect species, whereas cysteine and aspartic proteases appear to be distributed mainly in Coleoptera. Since the assimilation of dietary proteins is critical to insect survival, inhibition of digestive proteases with PIs presents itself as a possible target for development of effective strategies for insect control.

A number of plant PI genes have been cloned, reconstructed and expressed in transgenic plants (Jouanin et al. 1998; Schuler et al. 1998). Serine PI genes were shown to specifically increase resistance to important pests that use serine proteases for protein



digestion (Hilder et al. 1987). In addition, overexpression of a *Nicotiana alata* gene that is posttranslationally cleaved into five individual serine PIs in transgenic plants was shown to effectively target insects classified into four different insect orders (Heath et al. 1997). Several coleopteran pests that commonly use cysteine proteases for protein digestion, as well as nematodes, were inhibited by cysteine PI genes of the oryzacystatin I (*OCI*) and II (*OCII*) PI gene family (Abe and Arai 1985; Kondo et al. 1990; Michaud et al. 1995; Samac and Smigocki 2003; Urvin et al. 1995).

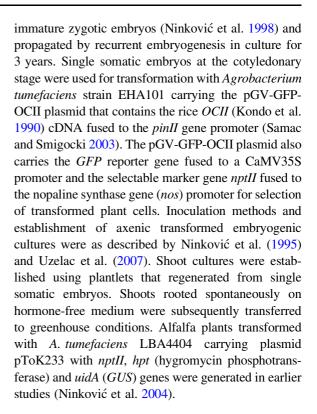
In this study, we expressed the OCII gene in alfalfa (Medicago sativa cv. Zaječarska 83) in order to evaluate its effects on Phytodecta fornicata Brüggemann (Coleoptera: Chrysomelidae, Lucerne leaf beetle) larvae. The Lucerne leaf beetle is an alfalfa field pest of the Balkan peninsula (Gradojević 1953). It is a monophagous herbivorous species that attacks only alfalfa and causes serious economical losses by defoliating the plants. While adult beetles feed only on the leaves, larvae will also consume petioles and young stems leading, in some cases, to complete crop loss. Although the Lucerne leaf beetle is not known to occur in other parts of the world, it has the potential for impacting North American agriculture since it has been consistently detected during the past 2 years on ceramic tiles imported from Italy (Source: USDA-APHIS). Malathion, a very toxic, non-systematic insecticide is being used to control this pest in alfalfa fields, but alternative, environmentally friendly control strategies are needed.

Characterization of *P. fornicata* digestive enzymes has not been reported, but we speculated that, as in other coleopteran pests (Murdock et al. 1987), the major midgut proteases likely fall into the cysteine class. In this study, we transformed alfalfa with the *OCII* gene in order to evaluate its effect on *P. fornicata*. We observed significant increases in mortality of second and third instars when they were fed transgenic alfalfa leaves that express the *OCII* gene.

Material and methods

Plant material

Initial embryogenic cultures of alfalfa (*Medicago* sativa L. cv. Zaječarska 83) were obtained from



Plant culture medium and conditions

Embryogenic cultures were maintained on a medium containing MS mineral salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 3% sucrose and 0.6% agar (*Torlak*, Belgrade, Serbia). Shoot cultures were propagated and rooted on hormone-free MS medium. The pH of the medium was adjusted to 5.8 prior to autoclaving at 114°C for 25 min. Cultures were grown in a temperature controlled chamber at 25 \pm 2°C under a 16/8 h photoperiod. Photosynthetic photon flux density of 31 μ mol m⁻² s⁻¹ was provided by white fluorescent tubes (*Tesla*, Pančevo, Serbia, 65 W, 4500 K).

PCR analysis

pGV-GFP-OCII transformed embryogenic cultures and regenerated shoots were analyzed by polymerase chain reaction (PCR; PE Applied Biosystems kit, Roche, New Jersey, USA). Plant DNA was isolated according to Zhou et al. (1994). PCR reactions were performed using gene specific primers to amplify a



800-bp fragment from the *pinII:OCII* construct. The following primer pairs were used: *pinII* forward 5′-GGC TCC TCC GTC CAA TTA TA-3′ and *OCII* reverse 5′-GGT GGC GTC GTC GAG GGG-3′. PCR reactions were initiated by denaturing at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, annealing at 53°C for 1 min and polymerization at 72°C for 2 min. PCR products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized with UV light.

Immunoblot analyses

Leaves of 1-year-old greenhouse grown plants were wounded with scissors. Each leaf was wounded by making two 0.5 cm long cuts on each side of the leaf. Wounded leaves were collected at 0, 4, 12, 24 and 48 h after wounding. Leaf proteins were extracted as described by Leple et al. (1995). Total proteins (50 µg per sample; Bradford 1976) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% acrylamide (Laemmli 1970) and transferred to polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich, Germany). The recombinant OCII protein was detected using alkaline phosphatase-conjugated anti-antibody system described by Blake et al. (1984) with cross-reacting (OCI and OCII proteins share 55-78% homology, Kondo et al. 1990) polyclonal OCI antibody provided by Dr. Lise Jouanin (INRA, Versai, France; Leple et al. 1995). Western blot analysis was repeated.

Insects

P. fornicata insects were collected in infested alfalfa fields in Batajnica (20 km north of Belgrade, Serbia) from the middle of March until the end of June in year 2001–2003. A colony of *P. fornicata* was maintained in a ventilated glass container in a temperature controlled chamber at $23 \pm 2^{\circ}$ C under an 16/8 h photoperiod. Insects were fed fresh alfalfa leaves daily. Larvae from offspring generation were used in feeding bioassays.

Feeding bioassays

Feeding bioassays were done with in vitro grown OCII transgenic shoots. Foliage were detopped from

3-week-old shoots and placed in a water-filled vase in a transparent plastic 200-ml cup. Second, third or fourth-instars were added to each cup and foliage was changed every second day. The number of dead larvae was recorded daily during 5 days of the instar. Assay consisted of six replicates with five larvae per cup. Experiments were repeated three times.

Statistical methods

The comparison of survival curves between larvae reared on different plants (control or transgenic) was carried out by χ^2 test. PHREG procedure (SAS, 6.07) was used to test for overall differences in surviving probability within each larval instar. Replicates were pooled within host plants. Therefore, sample size was 30 within each experimental group.

Results

Plant transformation

From the 200 embryos that were infected with A. tumefaciens strain carrying the pGV-GFP-OCII binary transformation vector, 28 embryogenic clones survived the kanamycin antibiotic selection pressure conferred by the nptII gene. These clones continued to grow on 50 mg/l kanamycin for at least 6 months in culture. Plants were regenerated from 11 of the 28 lines (OCII-1, -2, -3, -4, -5, -7, -8, -12, -15, -16 and -19). In general, transformed plants elongated slowly in comparison to the control plants. Two of the best growing shoot lines, OCII-2 and -15, were analyzed by PCR, using a forward primer specific for the pinII promoter and a reverse primer for the OCII gene. DNA fragments corresponding in size to those expected for the pinII-OCII gene construct were detected (Fig. 1; Samac and Smigocki 2003).

OCII expression in transformed alfalfa plants

OCII protein accumulation was demonstrated in leaves of greenhouse grown OCII-2 transgenic plant after mechanical wounding of the leaves (Fig. 2). A 12 kDa peptide was detected in transgenic leaf samples collected 24 h and 48 h after wounding



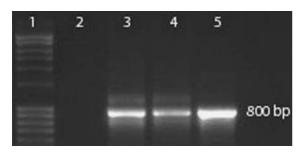


Fig. 1 PCR analysis of two alfalfa lines transformed with *OCII* gene. Primer set used in this analysis amplified a 800 bp fragment from the *pinII:OCII construct*. Lane 1—100 bp DNA Ladder; lane 2—untransformed control; lane 3—OCII-2 shoots; lane 4—OCII-15 shoots; lane 5—plasmid transformation vector, AgV-GFP-OCII-4.2A-7

(Fig. 2, lane 4 and 5). No cross-reactivity of the OCI antibody with endogenous alfalfa cystatins was observed in control plants either before or after wounding (Fig. 2, lanes 6–10).

Insect bioassay of OCII transgenic plants

Due to the difficulties in obtaining large numbers of acclimated plants that express the *OCII* transgene, feeding bioassay was performed on in vitro grown shoots. OCII-2 and -15 lines were used to test for resistance to *P. fornicata* larvae.

Significant differences in larval survival were observed when second instars fed on leaf material from the OCII transgenic plants as compared to the control (Fig. 3, OCII-2 χ^2 = 14.859, P < 0.000; OCII-15 χ^2 = 10.249, P < 0.001). About 70% of the larvae reared on OCII and 0% on control shoots were dead on day 1 (Fig. 3). Mortality increased to 80% on OCII shoots on day 2 but remained close to 0% on control shoots. Mortality of second instars reared on control shoots increased markedly on day 3 but all larvae that survived entered the pupal stage.

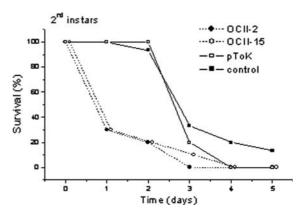


Fig. 3 Survival of second instars fed on transgenic OCII-2 and -15; transformed control (pToK); and untransformed (control) shoots. Sample size was 30 within each experimental group

Similar differences in larval survival were also observed when third instars were reared on the control and transformed OCII shoots (Fig. 4, OCII-2 $\chi^2 = 13.087$, P < 0.000; OCII-15 $\chi^2 = 9.957$, P < 0.001). The mortality of third instars fed on OCII transgenic shoots on day 2 was about 80%, similar to what we observed with the second instars. Such high reduction in survival was not observed on day 1 in third instars as with the second instars that fed on the OCII transgenic shoots.

There were no significant differences in survival between fourth instars fed on either the control or transgenic plant material (Fig. 5, OCII-2 χ^2 = 0.011, P < 0.915; OCII-15 χ^2 = 1.649, P < 0.199). The mortality of fourth instars on day 2 was about 20%, four time less than what we observed with the second and third instars fed on OCII-2 and OCII-15 transgenic shoots on the same day. Similarly, larval survival on untransformed, control plants lacking the *OCII* gene (pToK) was not statistically different from untransformed (control) plants for any of larval stages tested (Fig. 3, second— χ^2 = 0.026, P < 0.871; Fig. 4, third— χ^2 = 0.004, P < 0.947;



Fig. 2 Immunoblot detection of OCII protein in leaf extracts from wounded transformed alfalfa plant (line OCII-2). Total protein extracts (50 μg) were loaded per lane. Lane 1–5, leaf

samples from greenhouse-grown OCII-2 plant at 0, 4, 12, 24 and 48 h after wounding, respectively; lane 6–10, control plant at 0, 4, 12, 24 and 48 h after wounding, respectively



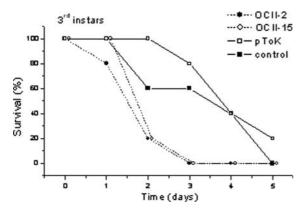


Fig. 4 Survival of third instars fed on transgenic OCII-2 and -15; transformed control (pToK); and untransformed (control) shoots. Sample size was 30 within each experimental group

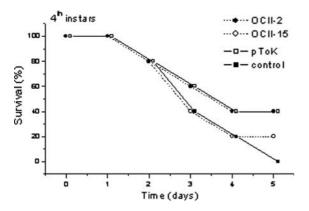


Fig. 5 Survival of fourth instars fed on transgenic OCII-2 and -15; transformed control (pToK); and untransformed (control) shoots. Sample size was 30 within each experimental group

Fig. 5, fourth— $\chi^2 = 0.011$, P < 0.915). It means that transformation with pToK233 plasmid did not affect larval survival.

Discussion

The Lucerne leaf beetle (*P. fornicata*) is one of the most serious pests of alfalfa in the Balkan areas of Europe. Therefore, in our study, we tested the effects of *OCII* transgene expression on the mortality of this insect. In order to minimize the exposure of nontarget organisms to the defense gene, conserve the metabolic energy of plants and minimize physiological effects of the transgene on plant metabolism, the *OCII* gene was fused to a promoter that is inducible

by wounding and insect feeding (pinII) (Samac and Smigocki 2003). A high degree of insect resistance in transgenic plants was evidenced in our bioassay. Deleterious effects on second and third instars feeding on alfalfa plants that express the OCII gene were observed. These effects coincided with the observed detection of the OCII protein in leaves of wounded transgenic plants (Fig. 2). The high mortality rate of second instars after 1 day of feeding on transgenic plants is associated with their overall smaller size (3-5 mm, Gradojević 1953) as compared to third (5–7 mm) and fourth (7–10 mm) instars. This difference in size is speculated to be primarily responsible for the observed 1 day delay in mortality of third instars when compared to second instars. No significant difference in survival curves was observed with the fourth instars indicating that the efficiency with which OCII inhibits *P. fornicata* appears to be dependent on the developmental stage and, therefore, size of the larvae. Using stronger promoters for generating higher in planta levels of OCII might be an effective strategy for targeting the older larvae.

Transformation of alfalfa with genes conferring insect resistance has been previously reported (Strizhov et al. 1996; Thomas et al. 1994). Strizhov et al. (1996) reported resistance against two lepidopteran species that was mediated by the cryIC transgene. Thomas et al. (1994) showed that a Manduca sexta anti-elastase gene reduced the number of Frankliniella spp. insects on alfalfa. Although a tomato serine protease inhibitor (proteinase inhibitor I) gene was expressed in alfalfa, no information regarding its effect on insect feeding was presented (Narvaez-Vasquez 1992). In this report, we document for the first time the transformation of alfalfa with a plant derived PI gene that significantly reduced the survival of P. fornicata second and third instar larvae. Results obtained in our study may be useful for developing alternative to classical P. fornicata management strategies for this devastating insect pest of alfalfa.

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